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Methylations in human hemoglobin

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Summary

Levels of *N*-Methylvaline (MeVal) and N^{τ}-methylhistidine (MeHis) were measured in male smokers and non-smokers in a program aimed at mapping background alkylations of hemoglobin (Hb) as potential indicators of doses of exogenous and endogenous genotoxic agents. MeVal was also determined in Hb from rats, Syrian golden hamsters, mice and chickens.

MeVal was found to occur at levels around 0.5 nmole/g Hb, with relatively little variation between individuals and species. MeVal was not significantly affected by smoking. This result contrasts with elevated levels of *N*-hydroxyethylvaline (HOEtVal) measured in the same persons (Törnqvist et al., 1986b). Levels of *S*-methylcysteine (MeCys) (Bailey et al., 1981) and MeHis were much higher than those of MeVal. The high levels of MeCys and MeHis may be due partly to misincorporation during protein synthesis and to artifacts. *S*-Adenosylmethionine and formaldehyde are possible endogenous sources of MeVal.

One individual (smoker) out of 21 selected for measurement of MeVal was an outlier, with raised levels of both MeVal and HOEtVal, as would be expected in case of a defective detoxification system.

Most ultimate mutagens and cancer initiators are electrophilic reagents (Miller and Miller, 1966) that react with nucleophilic atoms not only in the supposed target molecule, DNA, but also in cellular proteins etc. Hemoglobin (Hb) was proposed for use as a monitor of mutagens and carcinogens because of its general availability and its long life span which permits the determination of cumulative doses (Osterman-Golkar et al., 1976). Due to proportionality between levels of DNA adducts and protein adducts the demonstration of Hb adducts is thus a relevant measure of the formation of the corresponding DNA adducts (Ehrenberg and Osterman-Golkar, 1980; Neumann, 1983). Furthermore, since dose–response curves may be assumed to be linear the demonstration of Hb adducts implies the identification of potential genotoxic risk. For a quantitation of this risk

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Abbreviations: Hb, hemoglobin; MeVal, *N*-methylvaline; MeCys, *S*-methylcysteine; MeHis, N^{τ}-methylhistidine; HOEtVal, *N*-(2-hydroxyethyl)valine; PFPTH, pentafluorophenylthiohydantoin; SAM, *S*-adenosylmethionine; MMS, methyl methanesulfonate; HFB-, heptafluorobutyryl-; GC/MS, gas chromatography/mass spectrometry.

additional information is required, however, comprising primarily identification of the causative electrophile and its source as well as establishment of the DNA/Hb adduct ratios for relevant target organs (Ehrenberg et al., 1983).

The measurement of Hb adducts provides a basis for monitoring tissue doses (Ehrenberg et al., 1983) of known environmental factors such as occupational exposures (Calleman et al., 1978; van Sittert et al., 1985; Osterman-Golkar et al., 1984), tobacco smoking (Törnqvist et al., 1986b) and use of alcohol (Stevens et al., 1981). A major additional application of this approach resides in its potential ability to identify exposures to a priori unknown mutagens/cancer initiators. Thus, a new method, developed to specifically cleave off alkylated *N*-terminal amino acids (Törnqvist et al., 1986a), permits the identification by gas chromatography–mass spectrometry (GC/MS) of several adducts in one and the same sample. This method should, therefore, prove useful in a search for unknowns and has been applied in the current study.

This paper reports the results of a study to determine the levels of *N*-methylvaline (MeVal) in the Hb of smokers and non-smokers. N^{τ}-Methylhistidine (MeHis) was also measured in a few of the samples. Methylations of the *N*-terminus of other proteins have been described previously (Chang, 1978), as well as of cysteine (MeCys) in Hb (Bailey et al., 1981). Determination of the ratios of specific amino acid adducts may provide information on the nature of the alkylating species (Svensson and Osterman-Golkar, 1986; Segerbäck, 1985). Since Bailey et al. (1981) found large variations between vertebrate species in the levels of MeCys in Hb, the inter-species variation in levels of background MeVal was also studied.

The human Hb samples used in this study originated from a well-defined group of smokers and non-smokers and had previously been used to measure *N*-hydroxyethylvaline (HOEtVal) content (Törnqvist et al., 1986b).

Materials and methods

Blood samples

The human blood samples analyzed in this study had been taken from 2 groups of healthy male subjects, all of age 56, of normal body weight, blood pressure, liver function, etc., and of comparable profession, polarized only with respect to smoking — non-smokers and individuals who smoked more than 20 cigarettes/day. The samples were collected and hemoglobin was isolated as described by Törnqvist et al. (1986b). Essentially similar procedures were used to isolate hemoglobin from chicken, mouse, rat and Syrian golden hamster.

Quantitative analysis of methylvaline in Hb

Reference compounds. *N*-Methylvaline (MeVal) was obtained from Sigma Chemical Company (St. Louis, MO). The pentafluorophenylthiohydantoin (PFPTH) derivative of this amino acid was prepared according to Törnqvist et al. (1986a).

Synthesis of standard globins. [$^{12}C_1$,$^{2}H_3$]-Methyl iodide (99.95 atom % ^{12}C, 99.0 atom % ^{2}H; obtained from Sigma) was mixed with [^{14}C]methyl iodide (2.5 mCi/mmole; New England Nuclear, MA) to give a specific activity of 6.1 μCi/mmole (determined as described below). About 20 mg of this radiolabeled methyl iodide was added to fresh blood (5 ml) which was incubated for 2 h at 37°C and then left at room temperature overnight. The red cells were isolated by centrifugation and carefully washed with 0.9% NaCl. The cells were lysed with water. The hemolysate was dialyzed against water and the globin was precipitated as described by Segerbäck et al. (1978).

The same procedure was applied to prepare non-radiolabeled methylated globin using methyl methanesulfonate (MMS) as methylating agent.

Quantitation of MeVal in standard globins. The specific activity of the mixture of labeled methyl iodides was determined. This was done by reaction of the mixture with *N*-acetylcysteine, hydrolysis of the reaction product and isolation of the labeled MeCys through ion exchange chromatography on Dowex 50W-X4 (cf. Segerbäck et al., 1978). MeCys was then quantitated through amino acid analysis and the radioactivity determined using [^{14}C]toluene for internal standardization.

The degree of [$^2H_3$]methylation of *N*-terminal valine in the standard globin was determined by hydrolysis of a sample of the globin, and separation of the amino acids — with MeVal added as carrier — on an Aminex A-5 cation exchanger (0.9 cm × 46 cm). The column was developed with 50 mM sodium citrate, first with 15 ml pH 3.2 and then with pH 4.8 and fractions were collected. The temperature of the column was 50 °C. The flow rate, 60 ml/h, was maintained by a Chromatronic CMP-1 pump. MeVal eluted after 90 ml of the second buffer. The quantitation of the content of MeVal was then based on radioactivity determination of the specific activity measured as described above. The same degree of [$^2H_3$]methylation was found by determination of the PFPTH by gas chromatography–electron capture detection (GC–ECD) after derivatization of the standard globin with pentafluorophenyl isothiocyanate (according to Törnqvist et al., 1986b) and comparison with the PFPTH prepared from methylvaline (MeVal). The yield of the PFPTH prepared from the amino acid and the standard globin was assumed to be 100% (Törnqvist et al., 1986a) and 65% (Törnqvist et al., 1987), respectively. Counting of the radioactivity in the labeled PFPTH from the derivatized standard globin gave consistent results.

The degree of methylation of *N*-terminal valine of the unlabeled standard globin was based on GC–ECD determination of the respective PFPTH of derivatized methylated and [$^2H_3$]methylated standard globins. [$^2H_4$]Hydroxyethylated globin was used as internal standard (cf. Farmer et al., 1986).

The degree of alkylation at the *N*-terminal valine in the standard globins was determined to be 2.9 μmoles [$^2H_3$]MeVal/g Hb and 7.2 μmoles MeVal/g Hb, respectively.

Calibration curve for MeVal in the globin samples. 50 mg ribonuclease A (Sigma) was dissolved in 3 ml formamide and samples of the methylated and [$^2H_3$]methylated standard globins were added. The proteins were derivatized with pentafluorophenyl isothiocyanate as described above. The calibration curve was linear in the range of interest.

Isolation and derivatization of globin samples. Globin was precipitated and 50-mg portions were derivatized in formamide with pentafluorophenyl isothiocyanate as described by Törnqvist et al. (1986b). [$^2H_3$]Methylated and [$^2H_4$]hydroxyethylated (cf. Farmer et al., 1986) globins were used as internal standards at final concentrations of 0.6 and 1 nmole/g globin.

Quantitation of MeVal in globin samples. The analyses of samples were carried out using a Finnigan 4021 (with a 4500 ion source) GC/MS in the negative ion chemical ionization, multiple ion detection mode. The operating parameters for the mass spectrometer were as follows: methane reagent gas at an ion source pressure of 0.60 torr (80 Pa); ion source temperature 100 °C; ionization energy 70 eV. The operating parameters for the gas chromatograph were: helium carrier gas at a pressure of 10 psi (69 kPa); temperature programming: 1 min at 100 °C, 20 °C/min to 200 °C, then 10 °C/min to 320 °C. A 30 m DB-5 (0.33 mm ID, 1 μm phase thickness) fused silica capillary column was used (J and W Scientific, Inc., CA). 1 μl of the toluene solutions of the derivatized samples was injected directly (on-column injector OCI-3, Scientific Glass Engineering, Ltd., Australia). The PFPTHs of MeVal and its deuterated analogue were monitored at *m/z* 310 ($M - CH_2CH_2$) and 313, respectively, on which the quantitation was based. The *m/z* 337 and 340, respectively, (M − 1) and other important fragments (see Törnqvist et al., 1986a) were also monitored.

Quantitative analysis of MeHis in Hb

Radiolabeled N^τ-methylhistidine was prepared by incubating N^2-acetyl[^{14}C]histidine with *N*-methyl-*N*-nitrosourea in water at room temperature. The solution was evaporated to dryness and the product was hydrolyzed in 4 M HCl for 4 h at 90 °C. N^τ-Methylhistidine was isolated by ion exchange chromatography (cf. below). N^τ-Ethylhistidine was prepared as described by Murthy et al. (1984).

Hemolysates were dialyzed against buffer and the protein was then hydrolyzed by addition of enzymes (as described by Osterman-Golkar et al., 1984). A known amount of radiolabeled N^τ-methylhistidine (10000 dpm, 0.003 nmole) was added

as tracer and internal standard. The samples were then acidified by addition of 4 M HCl to precipitate heme, filtered and evaporated to dryness. MeHis was isolated from hydrolysates by ion exchange chromatography according to Osterman-Golkar et al. (1984). Quantitation of the protein was based on measurement of histidine.

One portion of the MeHis fraction was analyzed on a Durrum D-500 amino acid analyzer. A second portion containing the internal standard, N^{τ}-ethylhistidine, was derivatized by esterification and acylation with heptafluorobutanoic anhydride and analyzed by GC/MS according to methods similar to those previously described (Calleman et al., 1978).

The quantitations were based on the peak areas of the fragment $m/z = 320$ from N^{τ}-methylhistidine (corresponding to loss of CO_2CH_3) and the corresponding fragment $m/z = 334$ from N^{τ}-ethylhistidine. The calibration curve gave a straight line, and the content of MeHis was determined from the calibration line. Good reproducibility was obtained when the quantitations were based on the ratios between $m/z = 379$ (M^+) for the HFB-MeHis and $m/z = 334$ for the HFB-N^{τ}-ethylhistidine.

Animal experiments with formaldehyde

In current studies to investigate formaldehyde as a potential source of Hb methylation, [^{14}C]formaldehyde, 10 mCi/mmole, was injected into mice (CBA males, b.w. 27 g; 0.042 mmole CH_2O/kg b.w.), and blood was collected after 4 h, 1, 3, 15, 24 and 35 days. Globin was isolated and [^{14}C]MeVal was analyzed after hydrolysis of the protein and ion exchange chromatography of the hydrolysate (with carrier methylvaline) using procedures described by Segerbäck et al. (1978).

Results

The levels of MeVal and MeHis measured in the Hb of smokers and non-smokers are summarized in Table 1. Except for one outlier the MeVal values show only small variations and fall in the range 0.4–0.7 nmole/g Hb. The outlier was a smoker and gave a value of 1.7 nmoles MeVal/g Hb. This high value was confirmed in several reanalyses. When this value is excluded, the mean values for smokers and non-smokers are virtually the same.

TABLE 1

LEVELS OF MeVal AND MeHis IN HUMAN Hb

MeHis measured by GC/MS was checked in a few samples by amino acid analyzer (AA) determination.

Person No.	MeVal (nmoles/g Hb)	MeHis (nmoles/g Hb)	
		GC/MS	AA
Non-smokers			
21	0.59	12	11
22	0.58	42	
26	0.55	15	
29	0.63	32	
01	0.58		
02	0.44		
04	0.36		
05	0.56		
09	0.50		
12	0.38		
15	0.36		
19	0.37		
20	0.58		
Mean	0.50		
Smokers			
23	1.7	7.1	11
24	[a]	8.8	12
25	[a]	20	
27	[a]	6.7	
28	0.65	3.5	3.1
06	0.56		
07	0.57		
08	0.59		
14	0.37		
17	0.55		
18	0.48		
Mean (except No. 23)	0.54		

[a] Sample used up for MeHis determination.

The MeHis values are higher than those for MeVal and show a greater relative variation. The precision and accuracy of the GC/MS method used to assay MeHis was checked by amino acid analysis of a few of the samples. The two methods gave good agreement.

The MeVal levels in Hb from different vertebrate species are summarized in Table 2, together with data from Bailey et al. (1981) for

TABLE 2

LEVELS OF MeVal AND MeCys IN Hb FROM DIFFERENT SPECIES

Species	MeVal [a] (nmoles/g Hb)	MeCys [b] (nmoles/g Hb)
Man	0.4–0.7 (–1.7)	16 (range 13–34 [c])
Rat	0.5 (±0.2) [d]	100
Hamster (Syrian golden)	0.4 (±0.2) [d]	6
Mouse	0.4 (±0.2) [d]	24
Chicken	0.3 (±0.2) [d]	500

[a] This study.
[b] Rounded-off values from Bailey et al. (1981).
[c] 7 persons (P. Farmer, personal communication).
[d] Uncertainty range of determinations because of variable background noise.

MeCys levels in the same species.

In the formaldehyde study globin samples were pooled from blood collected 4 h, 1 and 3 days after treatment of the mice. The radioactivity associated with the MeVal fractions corresponded to 200 pmoles MeVal/g Hb per mmole CH_2O/kg b.w. injected.

Discussion

Measurement of MeVal

The quantitation of MeVal in Hb after derivatization to the PFPTH was reproducible provided certain precautions were taken. A linear calibration curve was obtained using calibration samples prepared from standard globins derivatized together with ribonuclease. The increments of the MS response due to additions of the synthetic methylated globin to a human Hb sample were found to follow the calibration curve. This result established the reliability of the calibration curve for the quantitation of MeVal in Hb samples. The derivatized samples were used to measure several valine adducts.

Possible sources

As discussed previously for 2-hydroxyethyl (Törnqvist et al., 1986b) and 2-oxoethyl groups (Svensson and Osterman-Golkar, 1986) the observation of simple alkyl groups does in many cases not provide unequivocal information on the structure of the causative electrophilic reagent. The methyl group provides a good illustration since a variety of endogenous and exogenous sources are possible. Among the former, natural biochemical methyl donors such as *S*-adenosylmethionine (SAM) and choline give rise to non-enzymatic alkylations (cf. Ross, 1962) and may, to the extent that they occur in the compartments of the monitoring macromolecules, contribute to the observed methylations. Non-enzymatic methylation by SAM has been demonstrated in vitro with proteins (Paik et al., 1975) and DNA (Barrows and Magee, 1982; Rydberg and Lindahl, 1982; Näslund et al., 1983).

Formaldehyde is rapidly converted to other components of the pool of 1-carbon compounds and could thus give rise to methylations for instance via SAM. It would also be conceivable that *N*-methylidenevaline, i.e. the Schiff base of the metabolic intermediate formaldehyde, hypothesizing a biochemical mechanism for reduction, could constitute a source of MeVal.

Methyl chloride provides an example of a possible exogenous source. This agent is a general pollutant of ambient air originating from smoldering combustion of vegetation and from the ocean (Lovelock, 1974, 1975; Singh et al., 1979) and occurs also in tobacco smoke (Brunnemann et al., 1978). (Methyl chloride is also formed in the body through reaction of, e.g., SAM with Cl^-.) Dimethylnitrosamine is another example. This compound occurs in tobacco smoke (Stehlik et al., 1982) and also in foods and beverages (Preussmann, 1984).

Reaction-kinetic considerations

The inter-species variation in the MeCys content of Hb (Table 2) is partly due to differences in nucleophilic reactivity of cysteine-*S*. In a comparative study, the relative reactivities of cysteine in the Hb of man, mouse and rat towards ethylene oxide were found to be approximately 1 : 10 : 150 (Segerbäck, 1985). This result is consistent with the higher (5–10-fold) degree of methylation of this residue in the rat than in the mouse treated in vivo with an equal dose of methyl methanesulfonate (cf. data from Bailey et al., 1981, and Segerbäck et al., 1978). The observed difference between the background levels of MeCys in the

rat and mouse is, therefore, compatible with the view that this adduct originates, at least in part, from in vivo alkylation. The reactivity of cysteine-*S* in chicken Hb is indicated to be high (Hamboeck et al., 1981).

Both MeHis and MeCys were more abundant than MeVal in the current study and in earlier studies in experimental species (Tables 1 and 2). Since nucleophilic reactivity decreases in the order Cys-*S* > Val-*N* > His-*N*, the picture is not compatible with *one* methylator as the predominant cause of the observed Hb adducts. This impression is strengthened by the absence of a correlation between the levels of MeHis and MeVal in the human samples (Table 1; correlation coefficient $r = -0.4$, 4 *df*, $P > 0.1$). In a study of hydroxyethylated amino acids it was found that alkylated Cys and His but not Val are misincorporated during protein biosynthesis (Kautiainen et al., 1986). Since both MeCys (Thompson et al., 1956) and MeHis (Hardy and Perry, 1969) are normal constituents of proteins in foods and feeds, misincorporation of these amino acids in the biosynthesis of Hb may contribute to the background levels found. On the other hand, MeVal is not expected to be misincorporated (Kautiainen et al., 1986). Furthermore, MeVal is not formed as an artifact under the mild conditions of analysis (cf. Calleman et al., 1979) and it may, therefore, be concluded that the measurement of MeVal reflects the true alkylation rates in the body.

A reaction-kinetic study has shown that SAM has approximately the same reaction pattern as MMS, although with about 100 times lower reactivity (Näslund et al., 1983). Assuming the concentration of SAM in erythrocytes to be about 30 μM, a value found in human blood serum (Salvatore et al., 1971), this reagent would account for the observed MeVal levels (see Appendix 1). It would therefore be of interest to determine the concentration of SAM in erythrocytes. Increased urinary excretion of SAM, possibly reflecting high plasma levels, occurs in certain metabolic disorders (Applegarth et al., 1971). Information on the kinetic properties and cellular occurrence of choline would also be valuable in determining whether or not this compound may contribute to the background methylations.

It has been discussed whether non-enzymatic methylation of DNA observed after exposure to toxic agents could be caused by SAM (Barrows and Magee, 1982; Becker et al., 1981). The fact that MeVal levels, in contrast to HOEtVal levels (Fig. 1), could not be shown to be correlated with smoking suggests — provided that SAM is at all responsible for the methylations — that smoking exerts little influence on the metabolism of SAM.

Environmental concentrations of methyl chloride are too low for this compound to make a significant contribution to the observed levels of MeVal (see Appendix 1). The absence of a correlation between MeVal levels and smoking is consistent with this conclusion, i.e., that methylators in the smoke (methyl chloride, dimethylnitrosamine) could only give rise to an insignificant fraction of the observed MeVal levels.

The demonstration that formaldehyde gives rise to MeVal in Hb in vivo indicates that this reagent may make a significant contribution to the level of the adduct especially when the high concentration, 0.1–0.2 mM, of formaldehyde in tissues is considered (Consensus Workshop, 1984). Further studies are required in order to clarify to what extent this contribution is brought about via natural methylators such as SAM.

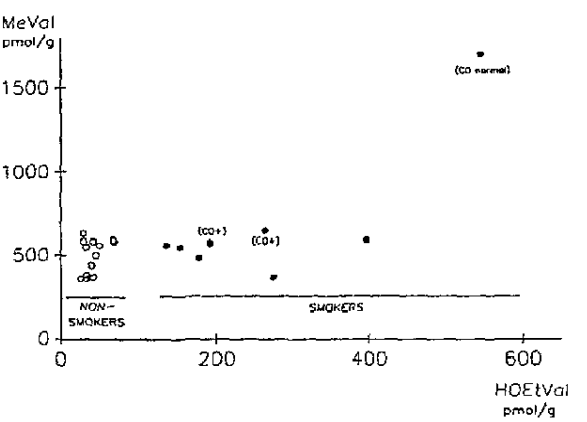

Fig. 1. Levels of *N*-methylvaline (MeVal) and *N*-hydroxyethylvaline (HOEtVal) in non-smokers (○) and smokers (●). CO+ indicates that the rate of CO exhalation at the time of sampling was > mean+2 S.D. —, in the previous study of the same samples (Törnqvist et al., 1986b) the MS readings for HOEtVal were adjusted to a non-linear calibration curve. It was later found that the calibration curve is linear, and in this figure the values have been re-adjusted accordingly.

Relations to risk

A risk estimation of methylating agents has to consider the efficient DNA-repair systems developed by living organisms (Näslund et al., 1983) and variations in the dose between organs as shown for methyl bromide (Djalali-Behzad et al., 1981).

Irrespectively of whether the sources of observed MeVal in Hb are endogenous or exogenous, the findings indicate the occurrence of a factor that is associated with risks of genotoxic effects. It is therefore important to clarify the origins of the observed methylations.

The observed MeVal levels show a tendency to be centered in two groups around 0.4 and 0.6 nmole/g Hb. This tendency is supported by the results of ongoing studies using a larger population (M. Törnqvist, current work) and may indicate a constitutive, possibly hereditary, variation in a detoxification function. The single high value, around 2 nmoles MeVal/g Hb, might be a consequence of a deficiency in such a function (cf. Vahakangas et al., 1984). The person giving this high value, although a normal smoker as judged from CO exhalation rate, also gave a relatively high result with respect to the HOEtVal level (Fig. 1).

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Appendix 1

Calculations of expected MeVal levels

After acute exposure the degree of alkylation ([RY]/[Y]) of a nucleophilic group (Y) in tissues is directly proportional to the dose (D) of the alkylating agent (RX) according to

$$[RY]/[Y] = k_y \cdot D$$

where $D = \int_t [RX]dt$ and k_y is the second-order rate constant for the reaction of RX with Y.

Due to the long life span of erythrocytes (~ 126 days in man) stable reaction products in Hb accumulate during continuous or intermittent exposure reaching a steady-state level of about 63 times the average daily increment of the alkylation (a) (Osterman-Golkar et al., 1976):

$$([RY]/[Y])_{acc} = 63 \cdot a$$

SAM. SAM is about 10^2 times less reactive than MMS (Näslund et al., 1983). The rate constant for reaction of MMS with *N*-terminal valine of human Hb is $0.6 \cdot 10^{-4}$ l (g Hb)$^{-1}$ h^{-1} (D.

Segerbäck, personal communication). The concentration of SAM in human blood serum is about 30 μmoles/l (Salvatore et al., 1971) and in various mammalian organs about 50 μmoles/l.

A 30 μM concentration of SAM in the red cells would give an accumulated level of MeVal of 27 nmoles/g Hb according to

$$([RY]/[Y])_{acc} = 63\ (\text{days}) \cdot 24\ (\text{h day}^{-1}) \cdot 30 \cdot 10^{-6}\ (\text{moles} \cdot \text{l}^{-1}) \cdot 0.6 \cdot 10^{-6} |\text{l (g Hb)}^{-1}\ \text{h}^{-1}|$$

$$= 27\ \text{nmoles (g Hb)}^{-1}$$

Methyl chloride. The kinetic constants for metabolism of inhaled methyl chloride in the rat have been studied using gas uptake measurements (Andersen et al., 1980). At low concentrations ($\ll K_m = 630$ ppm) the uptake corresponds to ~ 2% of the methyl chloride content in the breathing volume.

The methyl chloride content in the main stream smoke is ~ 0.65 mg/cig. (might give ~ 100 ppm in the breathing zone). Smoking of 30 cig./day, assuming a 2% uptake, an average body weight of 70 kg and a rate of elimination of $c \cdot 5\ \text{h}^{-1}$ (the rate found for methyl bromide in the mouse, Djalali-Behzad et al., 1981), would lead to a daily uptake of $0.11 \cdot 10^{-6}$ moles (kg b.w.)$^{-1}$ and a daily dose of $0.022 \cdot 10^{-6}$ M h *.

The reactivity of methyl chloride is ~ 200 times lower than that of MMS and the rate constant for reaction with *N*-terminal valine of Hb may thus be estimated as $0.3 \cdot 10^{-6}$ l (g Hb)$^{-1}$ h^{-1} i.e.

$$([RY]/[Y])_{acc} = 63\ (\text{days}) \cdot 0.022 \cdot 10^{-6}\ (\text{M h day}^{-1}) \cdot 0.3 \cdot 10^{-6}\ \left(\text{l (g Hb)}^{-1}\ \text{h}^{-1}\right)$$

$$= 0.42\ \text{pmole (g Hb)}^{-1}$$

SAM may also give rise to methylations via methyl chloride formed in alkylation of chloride (Cl^-). If $[SAM] = 30 \cdot 10^{-6}$ M, $[Cl^-] \approx 0.2$ M and $k_{Cl} = 10^{-3}\ \text{M}^{-1}\ \text{h}^{-1}$ (interpolated value from Näslund et al. (1983) using $n_{Cl} = 3.0$ (Koskikallio, 1969)) we obtain the rate of methyl chloride formation (v)

$$v = 10^{-3} \cdot 30 \cdot 10^{-6} \cdot 0.2 \cdot 24 = 144 \cdot 10^{-9} = 0.14 \cdot 10^{-6}\ \text{M day}^{-1}$$

This results in a daily dose which is approximately as large as the uptake by smokers. If we disregard exhalation of methyl chloride we obtain an upper limit of approximately 0.5 pmole MeVal/g Hb.

In the general environment levels of methyl chloride are about 1 ppb (Singh et al., 1979). The corresponding uptake will be negligibly low.

* $2\% \cdot \frac{30\ (\text{cig./day}) \cdot 0.65 \cdot 10^{-3}\ (\text{g/cig.})}{50\ (\text{g/mole}) \cdot 5\ (\text{h}^{-1}) \cdot 70\ (\text{kg})} = 0.022 \cdot 10^{-6}\ \text{moles kg}^{-1}\ \text{h day}^{-1}$.